university of groningen

University Medical Center Groningen

University of Groningen

On the mechanism of prokaryotic glutamate transporter homologues

Groeneveld, Maarten

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
2010

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):
Groeneveld, M. (2010). *On the mechanism of prokaryotic glutamate transporter homologues*. s.n.

Copyright
Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

The publication may also be distributed here under the terms of Article 25fa of the Dutch Copyright Act, indicated by the "Taverne" license. More information can be found on the University of Groningen website: https://www.rug.nl/library/open-access/self-archiving-pure/taverne-amendment.

Take-down policy
If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): http://www.rug.nl/research/portal. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.

Download date: 05-05-2023

Chapter 5

Biochemical characterisation of three prokaryal glutamate transporter homologues

Maarten Groeneveld, Ria Duurkens and Dirk Jan Slotboom

Abstract

Glutamate/aspartate transporters and their homologues (neutral amino acid and dicarboxylate transporters) form a large and widespread family of proteins that couple substrate transport to the cotransport of sodium ions and/or protons. The availability of a crystal structure from the homologous aspartate transporter Glt_{Ph} from the archaeon *Pyrococcus horikoshii* has been instrumental for the design of experiments to elucidate the transport mechanism of these proteins. Yet the experimental data on the functioning of the bacterial homologues of the family are limited and scattered, compared to data for eukaryal homologues. In this chapter we have characterised three bacterial glutamate transporter homologues, and emphasize both the differences and the similarities between prokaryal and eukaryal systems. Basic biochemical characteristics, such as substrate specificity, coupling ion dependency, temperature and pH dependency, and anion permeability were studied by using purified and reconstituted protein.

Introduction

Membrane proteins dedicated to transport of solutes across biological membranes can be classified on the basis of the mode of transport (94, 95). Transport can be passive via (facilitated) diffusion, or may be active requiring input of free energy from a coupled reaction. For instance the hydrolysis of ATP may provide the free energy to drive transport (as in primary active transporters). Alternatively, the coupled membrane transport of ions/solutes down their electrochemical gradients can provide the free energy to transport the substrate (as in secondary active transporters). The DAACS (dicarboxylate/amino acid:cation symporter) family forms a widespread group of secondary transporters in which the individual members differ both in substrate specificity and in the type of coupling ions used during transport. In higher eukarya these proteins play a role in glutamate-mediated neurotransmission (58). Glutamate is one of the major excitatory neurotransmitters (16), and its release in the synaptic cleft leads to continuation of a signal down the post-synaptic neuron. To prevent overexcitation of and concomitant damage to the neuron, a series of dedicated secondary transporters is present in the cell membranes of neurons and glial cells to rapidly remove glutamate from the synaptic cleft and maintain low extracellular glutamate concentrations. Glutamate transporter homologues from the DAACS family are found among higher eukaryotes, and are also encoded in most archaeal and bacterial genome sequences (102). Aspartate (9), dicarboxylates such as succinate and fumarate (54), and neutral amino acids such as serine, threonine (61) and putatively cystine (12) have been identified as substrates of different bacterial glutamate transporter homologues.

The crystal structure of the sodium coupled aspartate transporter from *Pyrococcus horikoshii* (121) showed that the protein is a homotrimer, which was also found for other members of the family, both in membranes and in detergent solution (36, 82, 120). Each protomer functions independently of the others (39, 41, 62, 63, 67), and displays a complicated membrane topology. An individual protomer is built up out of 8 transmembrane helices. Between helix 6 and 7, and 7 and 8, two reentrant regions are present, which play a crucial role in substrate binding and gating (10, 42, 43, 97, 103). The entire C-terminal end of the protein, consisting of transmembrane helix 7 and 8, and the two reentrant regions, forms a plug-like structure in a ring formed by the other 6 transmembrane helices (121). The large body of electrophysiological and biochemical data on eukaryotic and prokaryotic glutamate transporters, in combination with several crystal structures from Glt_{Ph} have resulted in a general model for the transport mechanism in glutamate transporters (14, 87). The protein utilizes the energy stored in the gradients of either sodium ions or protons to drive transport (symport). After initial cation and substrate binding, the substrate-binding pocket is shielded from the external solution by

binding of at least another cation followed by motion of the extracellular reentrant region towards the substrate. The protein then undergoes a large conformational change and substrate and cations are released to the internal space. Finally the empty transporter is reset in the conformation with the substrate binding site facing outward (14, 87).

In spite of the general mechanism that is likely conserved among the various homologues, there is a striking variation with respect to the type and number of coupling ions, not only between homologous proteins from different species but also between paralogues. The different eukaryotic EAAT-variants (excitatory amino acid transporters) couple glutamate transport to the cotransport of three sodium ions and one proton (68, 125). To reset the protein to the outward facing conformation, countertransport of one potassium ion occurs. The bacterial glutamate transporters GltP and GltT from *Escherichia coli* and *Bacillus stearothermophilus* respectively couple substrate transport to at least two protons, although GltT may couple transport to both protons and sodium ions in native membranes of *B. stearothermophilus* (106). Besides GltP, *E. coli* has three other glutamate transporter homologues: SstT, which is a sodium coupled serine/threonine transporter (stoichiometry unknown) (61, 74), DctA, a dicarboxylate transporter, which is proton coupled (19) and is likely to couple dicarboxylate transport to symport of at least 3 protons (chapter 3), and YdjN, which is uncharacterised. The archaeal aspartate transporter Glt_{Ph} couples aspartate transport to 3 sodium ions (chapter 4). In contrast to the eukaryotic EAATs, Glt_{Ph} does not require countertransport of potassium ions to reset the empty transporter to the outward facing conformation (82, 90).

An unusual feature of eukaryotic glutamate transporters is their permeability for anions such as chloride and nitrate. The transport or conductance of chloride is uncoupled from the main transport event, and is triggered in the EAATs by the presence of sodium and glutamate (7, 29, 110). Actual transport of substrate is however not required to induce a chloride-associated current (29, 110). Chloride permeability is not restricted to eukaryal transporters; addition of chloride or nitrate in transport experiments with Glt_{Ph} and GltT from *B. stearotherophilus* has hinted at uncoupled permeability for anions in these proteins as well (91).

The available experimental data on the transport mechanism of prokaryotic glutamate transporters is limited compared to the data available for their eukaryotic counterparts and the *P. horikoshii* aspartate transporter. To fill up some of the gaps in our knowledge on bacterial glutamate transporter homologues we have studied two bacterial proton coupled glutamate transporters (GltT, *B. stearothermophilus* and GltP, *E. coli*), using purified proteins reconstituted into proteoliposomes. Furthermore, we also studied SstT from *E. coli*, which has been described previously as sodium coupled serine/threonine transporter (61, 74). In this chapter we aim to highlight both the similarities and differences between bacterial DAACS family members and their eukaryal counterparts.

Materials and methods

Chemicals

Restriction enzymes were purchased from F. Hoffmann-La Roche Ltd (Basel CH). [^{14}C]glutamate (1.85 Mbq/ml, 9.36 Gbq/mmol), [^{14}C]aspartate (1.85 Mbq/ml, 8.03 Gbq/mmol and l-[U-^{14}C]serine (1.85 Mbq/ml, 6.07 Gbq/mmol) were obtained from Amersham Bio-Sciences AB (Buckinghamshire UK). All other chemicals were of analytical grade and obtained from commercial resources.

DNA manipulations

The *sstT* gene was amplified from *Escherichia coli* MC1061 genomic DNA, using Phusion polymerase according to the manufacturer's instructions. Primers used in this reaction were designed in such a way that both a dedicated 5'- and 3'- terminal sequence were added, making the insert suitable for the LIC-procedure (35). The resulting plasmid for expression of SstT was named pBADNlic-SstT. The vectors were designed in such a way that an N-terminal deca-histidine tag, followed by a TEV protease cleavage site was added to the transport protein products. DNA sequencing confirmed that no unwanted mutations had occurred (Service XS, Leiden The Netherlands).

Expression and purification of glutamate transporters

GltT and GltP were produced from plasmid pHISIII-GltT and plasmid pHISIII-GltP (31), and for SstT plasmid pBADNlic-SstT was used. Glt_{Ph} was expressed from a construct used by Ryan and Mindell (91). Expression was performed in *Escherichia coli* MC1061 cells for all four proteins, grown in Luria broth at 37°C and shaken at 200 rpm. Ampicillin was added to the cultures to a final concentration of 100 μg/mL. At an optical density of 0.8 at 660 nm, L-arabinose was added to a final concentration of 0.01 mg/mL. Two hours after induction the cells were harvested (10,000 rcf, 10', 4°C, Beckman JLA 8.1000 rotor) and resuspended in 20 mM Tris-HCl (pH 8). DNAse (10 μg/mL), PMSF (phenylmethanesulfonyl fluoride, 200 μM) and $MgSO_4$ (1 mM) were added, and cells were passed once through a Constant Systems cell disrupter at 20 kPsi (Constant Systems Ltd. Daventry UK) cooled at 4°C. Unbroken cells and cell debris were pelleted (5000 rcf, 25', 4°C, Harrier 18/80 swing out centrifuge), and the supernatant was subjected to ultracentrifugation (150,000 rcf, 90', 4°C, Beckman 50.2ti rotor). Membrane pellets were resuspended in 20 mM Tris-HCl (pH 8), and stored at -80°C. The protein concentration in the membranes was determined using bradford reagent, with BSA as a standard.

All proteins except Glt_{Ph} were solubilized from membrane vesicles in buffer A (50 mM Tris-HCl (pH 8.0), 300 mM NaCl), containing 15 mM imidazole pH 8.0 and 1% n-dodecyl-β-D-maltopyranoside (DDM), at a final protein concentration of 5 mg/mL. After incubation on ice for 45', with occasional gentle shaking, the solution was centrifuged (267,000 rcf, 15', 4°C, Beckman TLA 100.4 rotor). Supernatants were incubated on a rotating platform for 60' at 4°C with nickel-sepharose slurry (Fast-flow, GE Healthcare, bed volume of 0.5 ml), pre-equilibrated with buffer A containing 15 mM imidazole pH 8.0. The mixture was loaded on a biorad Poly-Prep column and unbound protein was allowed to flow through. Columns were washed with 20 column volumes of buffer A, supplemented with 60 mM imidazole and 0.04 % DDM. Protein was eluted from the column in three fractions of 300, 500 and 500 μL respectively, using buffer A, supplemented with 500 mM imidazole pH 8.0 and 0.04% DDM. The second elution fraction from the affinity chromatography contained most of the purified protein and was loaded on a Superdex-200 gelfiltration column, using 20 mM potassium phosphate pH 7.0, 150 mM NaCl, 0.04% DDM as eluens. Glt_{Ph} was purified as described by Ryan and Mindell (91).

Reconstitution of purified protein

For reconstitution in liposomes, a 3:1 (w:w) mixture of *E. coli* total lipid extract and egg PC (Avanti polar lipids) was prepared. Liposomes were homogenized by extruding 9 times through a 400 nm pore size filter, and diluted in 50 mM potassium phosphate pH 7.0, to a final concentration of 4 mg/mL. Liposomes were destabilized by step-

wise addition of triton X-100 (10% w/v). The absorption at 540 nm was monitored, and addition was stopped when a value of 2/3rd of the maximum absorbance was reached (34). Protein was added in a 1:500 ratio (w:w, protein:lipid) for GltT, and 1:250 for GltP, Glt_{Ph} and SstT. The reconstitution mixtures were incubated at room temperature for 30', under gentle movement. Biobeads (34) were added (25 mg/mL), followed by another 30' incubation at room temperature. After addition of more biobeads (15 mg/mL), the temperature was lowered to 4°C, and the solution was incubated for another 60' under gentle movement. Extra Biobeads were added (19 mg/mL), followed by overnight incubation at 4°C. Subsequently, a final amount of biobeads was added (29 mg/mL), followed by 120' incubation at 4°C. Biobeads were removed, and proteoliposomes were subjected to ultracentrifugation (267,000 rcf, 4°C, 20', Beckman TLA 100.4 rotor). Pellets were washed once in buffer of interest, and resuspended to a final lipid concentration of 20 mg/mL. Proteoliposomes were subjected to 3 cycles of freezing and thawing using liquid nitrogen and subsequently stored in liquid nitrogen.

Transport assays

Proteoliposomes were thawed, extruded as described above and centrifuged (267,000 rcf, 4°C, 20', Beckman TLA 100.4 rotor). Proteoliposomes were resuspended in the internal (luminal) buffer to a protein concentration of 0.5 μg/μL. In transport assays, proteoliposomes were diluted 100x into 200 μL of external buffer (specified in the results section). All assays were performed at 30°C, except when otherwise indicated, and internal and external buffers were iso-osmotic. Transport reactions were stopped by addition of 2 mL of ice-cold 100 mM LiCl, followed by rapid filtration over BA-85 nitrocellulose filters and an additional wash step with lithium chloride. Levels of radioactivity were determined by addition of 2 mL of emulsifier scintillator plus liquid (Perkin Elmer, Waltham USA), and analysed in a Perkin Elmer Tricarb 2800 TR isotope counter.

Results

Glutamate transporters have strict and narrow substrate specificity

GltP is a proton coupled glutamate/aspartate symporter, while SstT is a sodium coupled serine/threonine transporter. GltT couples glutamate/aspartate transport to proton transport when expressed in *E. coli*, but the protein also uses sodium ions when present in native membranes of *B. stearothermophilus* (106). GltT, GltP and SstT can be produced in *E. coli* membranes using the Ara/pBAD expression system (44) with yields of ~0.5-2% of total membrane protein. The three proteins have been solubilized in the detergent DDM and purified and reconstituted in an active form into liposomes (31, 61, 82). We determined K_m and V_{max} values for glutamate transport by GltP and GltT and serine transport by SstT, using purified proteins reconstituted in proteoliposomes (table 1). The K_m value for glutamate transport by GltT (3.55 μM) corresponded well with the value reported previously using membrane vesicles from *Bacillus stearothermophilus* (47). The K_m value for serine transport by SstT (0.07 μM) was lower than reported by Kim *et al* using proteoliposomes with purified protein (0.82 μM) (61). The reason for the discrepancy might be attributed to differences in the methods: Kim *et al* used a different detergent to solubilize the protein (octyl glucoside), performed a one-step purification (affinity chromatography) whereas we used two steps (affinity chromatography and size exclusion chromatography), and used detergent dilution to reconstitute the protein, while we used

adsorption to polystyrene beads to remove the detergent. GltP had a similar K_m for glutamate as GltT, but a significantly higher V_{max} value (1.92 and 0.35 nmol*(mg protein)$^{-1}$*sec^{-1} respectively). Since GltT originates from an organism that grows at much higher temperatures than *E. coli* (the origin of GltP), it is not unlikely that at higher temperatures GltT has a higher V_{max}, while GltP becomes less active or even inactive. GltP also transported aspartate (K_m of 3.6 μM ±1.38).

protein	K_m (μM)	V_{max} (nmol*mg^{-1}*s^{-1})
SstT	0.07±0.004	0.34±0.01
GltT	3.55±1.26	0.35±0.08
GltP	5.5±0.82	1.92±0.10

Table 1: Apparent K_m and V_{max} values of glutamate transport by GltT and GltP and serine transport by SstT. Initial transport rates were determined in the presence of a proton or sodium ion electrochemical gradient as described in the legend to figure 1, with different substrate concentrations. Data were fitted in the program Origin. Values are the averages of three measurements, errors indicate standard deviations.

The substrate selectivity of the homologous sodium coupled aspartate transporter Glt_{Ph} from *Pyrococcus horikoshii* is very different. Glt_{Ph} transports aspartate with a K_m of 0.12 μM, but glutamate only with very low affinity (9, 82, 90).

We also determined the affinity of SstT for sodium ions at a serine concentration of 0.9 μM, which is far above the apparent K_m. The K_m of SstT for sodium ions was 0.20 mM ±0.01 mM with a Hill coefficient of 1.08 ±0.22 (averages of three measurements, errors indicate standard deviations). For comparison, a K_m for sodium ions of 2.0 mM ±0.2 mM was reported by Ryan et al for the homologous sodium coupled aspartate transporter Glt_{Ph}, also at substrate concentrations that were well above the K_m for aspartate. For Glt_{Ph} a Hill coefficient for sodium ions of 2.6 ±0.6 was found (90). This value is consistent with the Na^+ to aspartate stoichiometry of 3:1 that we determined (chapter 4). The Hill coefficient for sodium ions in the serine transporter SstT could indicate that only one sodium ion is transported per molecule of serine, as is the case for human neutral amino acid transporters (126).

To determine the substrate specificity of GltT, GltP and SstT, we conducted a series of inhibition experiments, measuring transport of radiolabeled substrates (glutamate and serine for GltP/GltT and SstT respectively) in the presence of a 75-fold excess of unlabeled potential inhibitor (figure 1). Of all tested amino acids, only the addition of unlabeled

glutamate and aspartate fully inhibited transport of [^{14}C]glutamate uptake by GltT and GltP. For SstT, the addition of serine and threonine fully blocked [^{14}C]serine transport, while the addition of a 75-fold excess of glutamine reduced initial rates with approximately 50%, suggesting a low affinity for glutamine of SstT. Next to the natural L-amino acids, a series of other putative substrates was tested. Transport by GltT and GltP was inhibited by D-aspartate, but not by D-glutamate, consistent with previous reports (106). The addition of cysteate also strongly inhibited glutamate transport by GltT and GltP (106). Transport of serine by SstT was not inhibited by the D-stereoisomer of serine, as reported previously (61), nor by the D-stereoisomer of threonine. Uptake of [^{14}C]serine was partially inhibited by the addition of o-phosphoserine (~50% decrease).

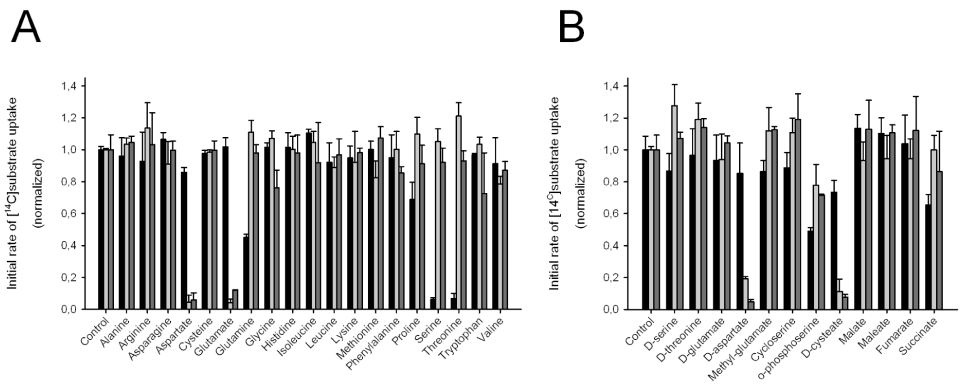

Figure 1: Substrate specificity of bacterial glutamate transporter homologues. Transport of [^{14}C]glutamate by GltT (dark grey), GltP (light grey), and [^{14}C]serine by SstT (black) was measured in the presence of a $\Delta\Psi$ (-118 mV) and a proton gradient (GltP/GltT) or sodium ion gradient (SstT). Proton and sodium ion electrochemical gradients were created by diluting proteoliposomes loaded with 100 mM K-HEPES (pH 7.5) 100 fold into external buffer (Na-MES pH 5.5 for GltP/GltT, and Na-MES pH 7.5 for SstT, both iso-osmotic), both containing valinomycin and radiolabeled substrate. The external buffer was supplemented with a 75-fold excess of the indicated compounds. Initial transport rates were determined and normalized to the rate without inhibitors. Measurements were done in triplicate, errors indicate standard deviations.

Temperature dependence of bacterial glutamate transporter homologues

The optimal growth temperatures for *E. coli* (from which GltP and SstT originate) and *B. stearothermophilus* (from which GltT originates) are very different (37°C and 60°C, respectively). We monitored the initial transport rates of glutamate (for GltP and GltT) and serine (for SstT) at temperatures between 20°C and 40°C. We were unable to obtain reproducible transport rates at temperatures higher than 40°C in our proteoliposomes, possibly because of leakiness of the proteoliposomes (109). Both SstT and GltT were hardly active at 20°C (figure 2A and C), but activity increased when raising the temperature. Q_{10} values over the entire measured range were 2.2 and 3.2 for GltT and SstT respectively. GltP

was significantly active at 20°C, and we determined a Q_{10} value of 2.2 over the entire measured range (figure 2B). These numbers indicate that considerable conformational changes take place in these proteins during a transport cycle.

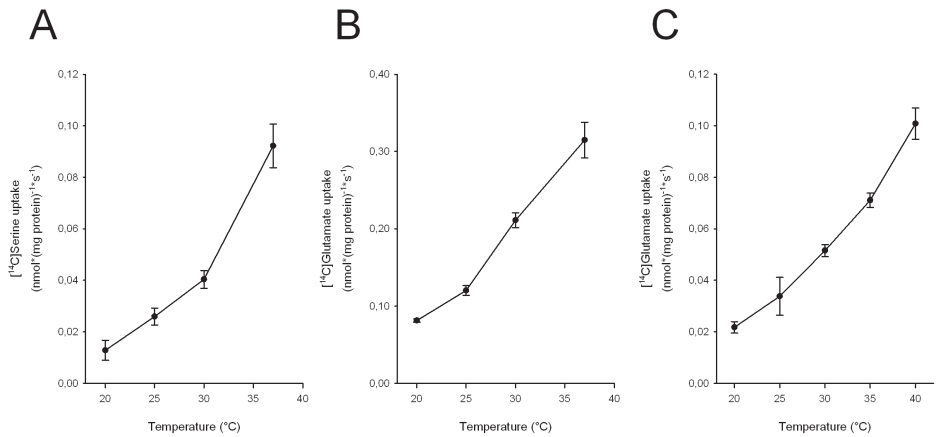

Figure 2: Temperature dependence of bacterial serine and glutamate transporters. Transport of [^{14}C]labeled substrate was monitored at different temperatures for SstT (A) GltP (B) and GltT (C) for their respective substrates, conditions as in figure 1. Measurements were done in triplicate and the error bars indicate standard deviations.

pH dependence of GltT

Eukaryotic glutamate transporters and the archaeal aspartate transporter Glt_{Ph} couple glutamate/aspartate transport to sodium ion symport. GltT and GltP in contrast do not use Na^+ coupling but instead use protons. To determine the pH dependence of glutamate transport by GltP and GltT, we monitored glutamate transport into proteoliposomes at different internal and external pH values. Interpretation of the pH dependence of glutamate transport by GltP/GltT is complicated because (1) protons are substrates (co-transported ions); (2) the protonation state of the amino acid substrates depends on the pH; (3) there may be other (regulatory) sites on the protein which can be protonated and of which the protonation state may affect activity. We performed several experiments to study the pH dependence of GltP and GltT, in addition we also performed similar experiments with the Na^+ coupled transporter SstT.

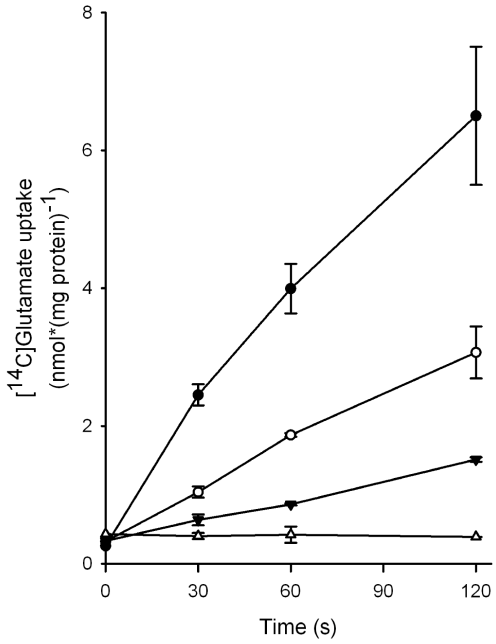

Figure 3: pH dependence of GltT. Proteoliposomes were loaded with a mixture of 50 mM K-MES/50 mM K-HEPES, set to pH 7.5, and transport was measured with an outside buffer composed of iso-osmotic Na-MES/Na-HEPES set to pH 5.5 (closed circles), 6.5 (open circles), 7.5 (closed triangles) or 8.5 (open triangles). External buffers contained 0.6 μM [^{14}C]glutamate and 0.5 μM valinomycin.

When the internal pH was kept constant at pH 7.5, we observed the highest initial uptake rate for GltT at the lowest external pH (pH 5.5) (figure 3). This observation is consistent with protons being co-substrates. Raising the internal pH from 7.5 to 8.5 at a constant external pH set to pH 5.5 did not significantly affect uptake rates (results not shown). When the pH gradient was kept constant at 2 units (outside acidic), transport rates were lower with an inside pH of 8.5, than at an internal pH of 7.5. This result contrasts with the pH dependence of $DctA_{Bs}$ (see chapter 3), and suggests that the rate limiting step for glutamate transport by GltT is the proton availability on the outside under these conditions.

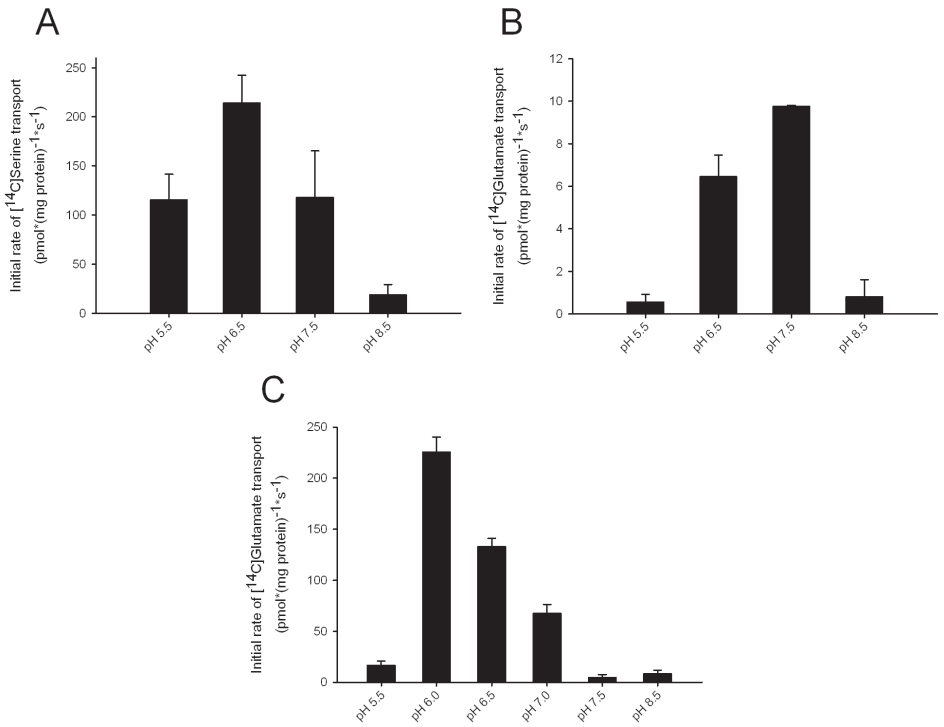

Figure 4: pH dependence of substrate transport. Proteoliposomes were loaded with a mixture of 50 mM K-MES/50 mM K-HEPES set to the pH indicated. Proteoliposomes were diluted in iso-osmotic Na-MES/Na-HEPES of the same pH, in presence of 0.5 μM valinomycin, and 0.9 μM [^{14}C]serine (SstT, panel A) or 0.6 μM [^{14}C]glutamate (GltT, panel B and GltP, panel C).

We also determined the transport rates of GltT and GltP when the internal and external pH values were equal, and accumulation was driven by an inside negative membrane potential (-118 mV). For comparison, we also did these experiments with the Na^+ coupled transporter SstT. Glutamate transport rates by GltP showed a sharp optimum at a pH of around 6, and the protein was almost inactive at pH values lower than 6 and higher than 7 (figure 4C). It is possible that at low pH values proton release on the inside becomes rate limiting, while at neutral and slightly basic pH values transport rates are limited by the external proton concentration (K_m for protons). Alternatively, GltP may become inactive at low pH because regulatory sites become protonated. Similar results were obtained for GltT, but GltT functions optimally between pH 6.5 and 7.5 (Figure 4B). The pH optimum for serine transport by SstT was much broader, with an optimum around 7 (figure 4A), as reported previously (61). SstT is still significantly active at a pH of 5.5, but loses most of its activity at a pH of 8.5. The activity of SstT reflected in figure 4A is a true pH optimum: protons are no substrate for this transporter.

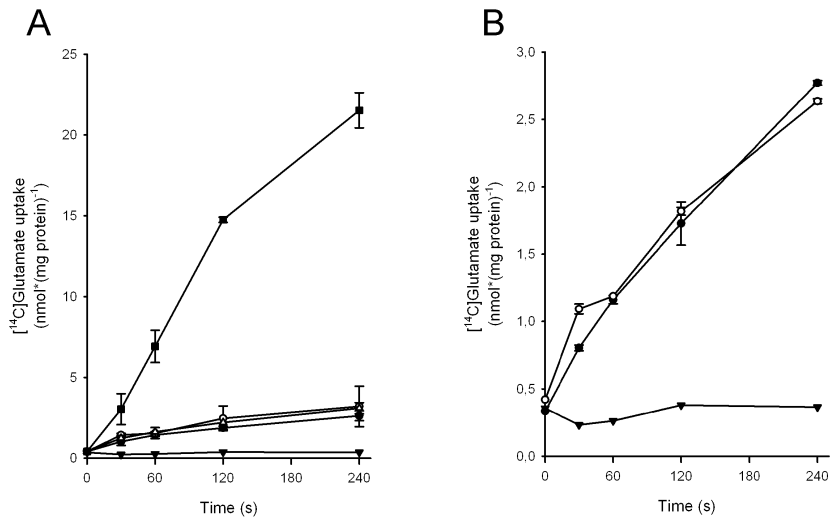

Figure 5: Potassium ion dependence of GltP. (A) Proteoliposomes were loaded with 100 mM K-HEPES (closed symbols) or Na-HEPES (open symbols) pH 7.5, and diluted in external buffer (iso-osmotic MeGluc-MES, pH 5.5) supplemented with 0.6 μM [^{14}C]glutamate. Valinomycin (0.5 μM) was added (closed squares, open triangles). (B) Proteoliposomes were loaded with 100 mM Na-HEPES, and diluted in iso-osmotic K-MES pH 5.5 (closed circles) or Na-MES pH 5.5 (open circles), supplemented with 0.6 μM [^{14}C]glutamate. As a control, measurements were done in absence of any gradients by diluting in 100 mM K-HEPES (pH 7.5) (closed triangles).

Potassium ion dependence of GltP and SstT

While eukaryotic transporters such as the EAATs are strictly potassium ion coupled, the sodium coupled aspartate transporter from *P. horikoshii* does not require potassium ions for substrate transport (82, 90). To establish whether the bacterial transporters GltP and SstT are also potassium ion independent, we first omitted potassium ions from the lumen of proteoliposomes harboring GltP. We observed glutamate transport levels that were equal to those in the presence of internal potassium (figure 5A). When we added a large excess of potassium to the external buffer, transport rates in the absence of valinomycin remained at similar levels as when no potassium was added to the external solution, which again suggests that GltP is not dependent on an outward facing potassium ion gradient to reset the transporter (figure 5B). When valinomycin was added, glutamate transport rates increased only in proteoliposomes that contained a higher internal than external concentration of potassium ions (figure 5A), because only in these proteoliposomes a negative inside membrane potential was generated. We performed comparable experiments for SstT, with similar results (figure 6). Initial transport rates for serine were almost equal in absence or presence of potassium ions.

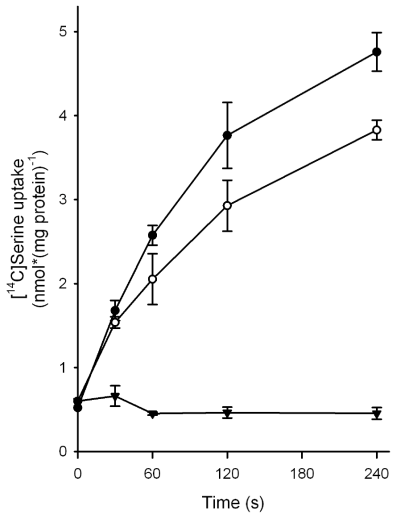

Figure 6: Potassium ion dependence of SstT. (A) Proteoliposomes were loaded with 100 mM K-HEPES (closed circles) or MeGluc-HEPES (open circles) pH 7.0. Samples were diluted in external buffer (100 mM Na-HEPES pH 7.0) supplemented with 0.9 μM [^{14}C]serine. As a control, measurements were done in absence of sodium, using external MeGluc-HEPES (closed triangles).

Magnitude of an applied membrane potential influences glutamate uptake in GltT

Bacterial glutamate transporters have been shown to be stimulated by the presence of an inside-negative membrane potential (e.g. figure 5A and (61, 106)). To further analyze the dependence of GltT on the membrane potential, we conducted experiments in which we made use of potassium ion gradients in combination with the ionophore valinomycin to impose potassium diffusion potentials of different magnitude and sign. Proteoliposomes harboring GltT were loaded with buffer supplemented with 10 mM potassium ions, after which the outside concentration of potassium ions was varied (figure 7). In this way, potassium diffusion potentials were created of different magnitudes, as indicated in figure 7. A negative membrane potential resulted in an increase of initial transport rates compared to transport in proteoliposomes in which the membrane potential was not fixed (absence of valinomycin). When the concentrations of potassium were equal on both sides of the membrane, and thus the membrane potential was set at 0 mV, addition of valinomycin also stimulated transport rates because it dissipated the charge build-up generated by glutamate-proton symport. Surprisingly, inversion of the membrane potential (positive inside) did not inhibit glutamate transport. The applied positive membrane potential might not be large enough to detect transport inhibition. A similar experiment was conducted for SstT, in presence of an inward facing sodium ion gradient. In contrast to

GltT, transport by SstT was inhibited by a positive membrane potential, using these specific conditions (data not shown).

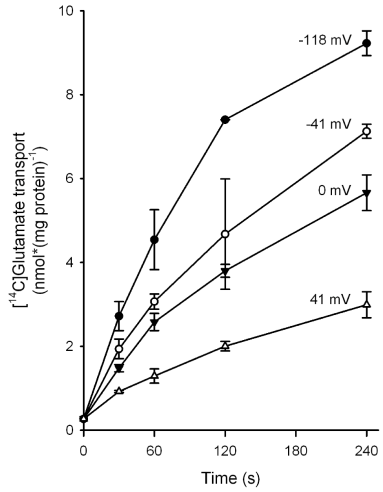

Figure 7: Glutamate transport by GltT in the presence of membrane potentials of different magnitudes. Proteoliposomes were loaded with 100 mM Na-HEPES (pH 7.5) supplemented with 10 mM KCl. Proteoliposomes were diluted in external buffer (Na-MES, pH 5.5, 0.6 μM [^{14}C]glutamate) supplemented with 0 mM KCl (closed circles), 2 mM KCl (open circles), 10 mM KCl (closed triangles) or 50 mM KCl (open triangles), all in the presence of 0.5 μM valinomycin. The concentration of Na-MES was adjusted to ensure that the external solution was iso-osmotic with the internal solution. The values of the K^+ equilibrium potentials are indicated.

Anion conductance by bacterial glutamate transporter homologues

Eukaryotic glutamate transporters display chloride conductance, which is uncoupled from the glutamate transport step. Ryan and Mindell have demonstrated for both Glt_{Ph} from *P. horikoshii* and GltT from *B. stearothermophilus* that such an uncoupled anion conductance can also be found in prokaryotic family members (90). In their experiments the electrogenic nature of Na^+-aspartate transport was exploited to study chloride conductance. Because asparate/glutamate transport is associated with net transport of positive charge into the liposome lumen, a positive membrane potential builds up upon import which impairs further transport. Clamping the membrane potential at more negative values than the equilibrium potential for glutamate transport prevents such impairment, leading to higher rates and accumulation levels (compare figure 7). When the membrane potential was clamped at -118 mV, a three fold increase in initial uptake rates was observed compared to the situation in which valinomycin was absent. Chloride gradients (concentration outside higher than inside) stimulated transport rates in Glt_{Ph} and GltT, indicating that the chloride ions also prevented build-up of a positive membrane potential. This observation led to the hypothesis that chloride conductance was present.

The presence of chloride resulted in a two-fold increase in transport rates, compared to conditions in which the non-permeant anion gluconate was present. The addition of other permeant anions also stimulated transport with nitrate showing the largest effect (three-fold increase in transport rates when compared to gluconate) (90).

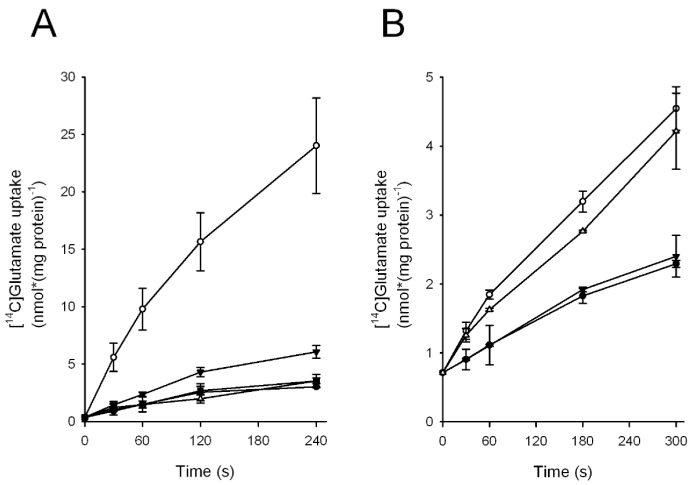

Figure 8: Effect of different anions on the initial transport rate of GltT. (A) Proteoliposomes were loaded with 100 mM K-HEPES (pH 7.5), and diluted into Na-MES (pH 5.5), supplemented with 0.6 μM [^{14}C]glutamate, and 25 mM of NaCl (open triangles), 25 mM NaGluconate (closed squares), 25 mM $NaNO_3$ (closed triangles) or no additional salt (closed circles). Valinomycin was added to generate a $\Delta\Psi$ (-118 mV, open circles). (B) Same as (A), but buffers were chosen in such a way that $[K^+_{in}] = [K^+_{out}]$. The external buffer was supplemented with chloride (closed triangles), gluconate (closed circles) or nitrate (open triangles). The $\Delta\Psi$ was clamped at 0 mV by addition of valinomycin (0.5 μM, open circles). In all cases external and internal solutions were iso-osmotic.

We repeated the experiments done by Ryan and Mindell for GltT, and extended the analysis to GltP and SstT, but we were unable to reproduce their results. In our experiments, no difference was observed in glutamate transport rates by GltT using gluconate or chloride in the external buffers (figure 8A). The addition of nitrate resulted in a small increase in activity, but not to the extent observed by Ryan and Mindell (90). When we repeated the experiment with equal potassium concentrations on both sides of the membrane, addition of chloride ions to the external buffer again did not result in an increase of initial rates, while addition of nitrate led to a small stimulation of transport (figure 8B). In the presence of valinomycin, thus clamping the membrane potential to 0 mV, an increase in initial transport rates was observed (figure 8B), similar to figure 7. We performed similar experiments for GltP and SstT, with comparable results (figure 9).

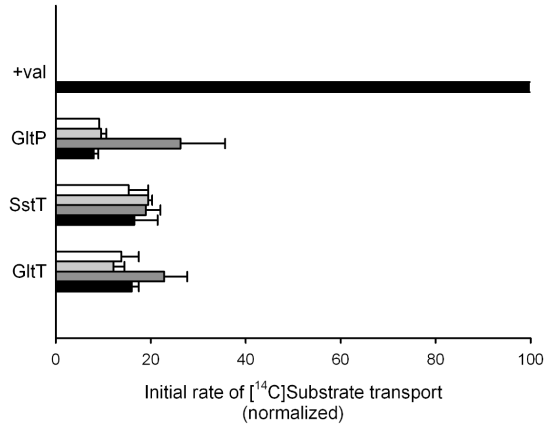

Figure 9: Effect of anions on the initial transport rates of GltP, SstT and GltT. Proteoliposomes were loaded with 100 mM K-HEPES (pH 7.5). Transport was driven by diluting the proteoliposomes in either Na-MES (pH 5.5, GltT and GltP), or Na-HEPES (pH 7.5, SstT), in the presence of 0.6 μM [^{14}C]glutamate or 0.9 μM [^{14}C]serine, respectively. External buffers were supplemented with 25 mM NaGluc (white), 25 mM NaCl (light grey) or 25 mM $NaNO_3$ (grey), or no additional salt (black). The Na-MES or Na-HEPES concentrations were varied to make the internal and external solutions iso-osmotic. Values were normalized to a transport experiment in which the membrane potential was clamped at -118 mV by the addition of valinomycin (0.5 μM).

We then attempted to reproduce the experiments conducted by Ryan and Mindell for the sodium coupled aspartate transporter Glt_{Ph} (90). We observed no difference between buffers to which gluconate or chloride was added as the anion (figure 10). Also, the three-fold increase in activity upon the addition of nitrate as seen by Ryan and Mindell could not be reproduced. We repeated these experiments under a range of conditions, and even visited the Mindell laboratory to ensure identical experimental conditions, but we were unable to measure apparent chloride conductance. At the moment we do not know what causes the differences between our results and those of Ryan and Mindell, and we must conclude that in our hands and under the conditions used we cannot demonstrate an uncoupled chloride conductance in prokaryotic glutamate transporters.

The addition of nitrate slightly stimulated the transport of substrate in the absence of a negative membrane potential (1.5-fold) (figure 10), but when the nitrate gradient was inverted (nitrate is included in the lumen of the proteoliposomes, but absent on the outside), transport was not inhibited (data not shown). When nitrate was added both to the external buffer and to the luminal buffer, transport was stimulated to a similar extent as when nitrate was present only on the outside. The observed stimulation by nitrate therefore seems to be related to an apparent effect of nitrate on the protein, and not by uncoupling of charge build-up.

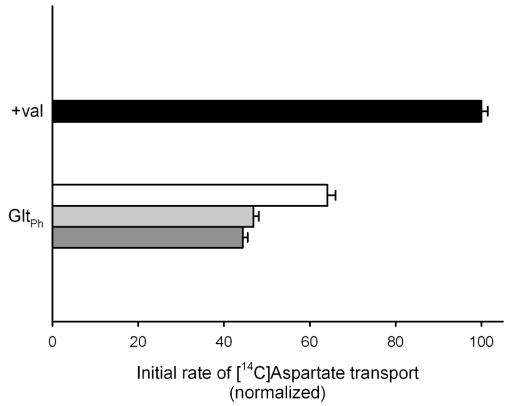

Figure 10: Effect of anions on the initial transport rate of Glt_{Ph}. Proteoliposomes were loaded with 100 mM K-HEPES (pH 7.5). Transport is driven by dilution of proteoliposomes in Na-HEPES (pH 7.5), in the presence of 0.7 μM [^{14}C]aspartate. External buffers were supplemented with 50 mM NaGluc (grey), 50 mM NaCl (light grey) or 50 mM $NaNO_3$ (white). Values were normalized to a transport experiment in which the membrane potential was clamped at -118 mV by the addition of valinomycin (0.5 μM).

Discussion

Glutamate transporters of the DAACS family are trimeric membrane proteins that are found throughout all kingdoms of life (102). These proteins use the energy stored in the gradients of protons and/or sodium ions to accumulate the amino acid substrate to high levels. In this chapter we studied transport by two bacterial glutamate transporters (GltP from *E. coli* and GltT from *B. stearothermophilus*), and the homologous serine transporter SstT from *E. coli*. These proteins are very selective for their respective substrates (figure 1A/B), and catalyze transport with high affinity (K_m values in the (sub)micromolar range). In contrast to their eukaryal counterparts, these proteins are not dependent on internal potassium ions to complete a transport cycle, but they are driven by proton symport (GltP/GltT) or sodium ion symport only (SstT) (figure 5/6).

Bacterial glutamate transporters have very narrow substrate specificity. As reported previously, the bacterial glutamate transporters GltT and GltP can transport L-glutamate and L-aspartate with high affinity (47, 106). Our finding that D-glutamate is not transported by these proteins while the addition of D-aspartate fully inhibits transport corresponds to previous reports in literature (47, 106). Of all the other putative substrates, only the addition of D-cysteate fully inhibited glutamate transport. Cysteate is an oxidized form of cysteine with a sulfonate group instead of a thiol group in the sidechain. None of the other compounds had a significant effect on glutamate uptake by either GltP or GltT. A similar narrow specificity was found for the threonine/serine transporter SstT from *E. coli*.

Transport of labeled serine was fully inhibited upon addition of an excess of unlabeled threonine or serine, as observed previously (61). Addition of glutamine and o-phosphoserine led to a 50% reduction of initial uptake rates; both compounds might be low affinity substrates for SstT. As reported previously by Kim et al (61), D-serine did not inhibit serine transport, nor did D-threonine. Our experiments do not distinguish between actual substrates and non-transportable inhibitors. Kainate and kainate derivates, and L-threo-β-benzyloxyaspartic acid (TBOA) are known examples of non-transportable inhibitors for eukaryal and prokaryal glutamate transporters (9, 16). Counterflow experiments as described in chapter 3 should be performed to confirm whether the found compounds are indeed transported.

In the *P. horikoshii* crystal structure, the substrate (aspartate) interacts with the protein via the alpha carboxylate and amino groups, and to a lesser extent via the side chain of the substrate (9). When our three bacterial proteins were aligned with the Glt_{Ph} amino acid sequence, a few differences were observed between SstT and the other proteins. The positive charge of the arginine at position 397 that interacts with the carboxylate group of the sidechain (*P. horikoshii* numbering) is replaced in SstT with a glutamate that possibly forms a hydrogen bond with the hydroxyl group of the substrates serine and threonine (9). The differences between Glt_{Ph} and the two glutamate transporters characterised in this chapter are minor. Most noticeable is the threonine at position 314 in the Glt_{Ph} sequence that is replaced by a serine in GltT and GltP. The presence of the smaller serine might make the binding pocket in GltT and GltP slightly larger, and thereby allowing glutamate, which is only a low affinity substrate for Glt_{Ph}, to fit in more easily.

The temperature dependence of the transport rates and the Q_{10} values found for all three proteins (>2) indicate that significant conformational changes are taking place upon substrate transport. These changes are most likely taking place within individual subunits, and not between adjacent protomers (39, 41, 62, 63, 67). Two recent papers including a new crystal structure of Glt_{Ph} indeed suggest significant movement in the protein during substrate translocation (14, 87), which could explain our observed Q_{10} values.

Bacterial glutamate transporters such as GltP, but also dicarboxylate transporters from the same family such as DctA from *Corynebacterium glutamicum* (122) and $DctA_{Bs}$ from *B. subtilis* (chapter 3) are strictly proton coupled, unlike eukaryal counterparts and Glt_{Ph}, which are driven by a sodium ion gradient. Our experiments confirm the dependence of GltT on a proton gradient. In the absence of a proton gradient, but in the presence of a negative membrane potential, low levels of transport were observed, imposing a proton gradient (outside acidic) had a stimulating effect on initial transport rates. Reversing the proton gradient diminished uptake rates to background levels. In our experiments, both GltT and GltP were fully independent of sodium ions (data not shown and figure 5). It has been reported previously that GltT, when reconstituted in *E. coli* lipids, is independent of

sodium ions (31). However, in membrane vesicles from *B. stearothermophilus*, GltT is reported to be dependent on sodium ions (106).

When the pH was equal on both sides of the membrane, GltP had a sharp optimum around pH 6 (figure 4C). The transport activity was almost completely lost by lowering the pH with half a unit, which cannot be explained by the protonation state of the substrate, since at pH 5.5 more than 90% of the glutamate is already in the double deprotonated state. No activity could be observed with pH values above 7.0. We performed most of the experiments with a pH gradient of 2 units, (internal pH of 7.5), because under these conditions transport rates were the highest. It seems that at acidic luminal pH values either the proton dissociation on the inside becomes rate limiting, or the protein itself is inactive. On the other hand the absolute amount of protons available on the outside becomes limiting at more neutral/basic pH (K_m for protons). Both SstT and GltT have a broader pH optimum (figure 4 A/B). GltT is active between values of 6.5 to 7.5, and is largely inactive at pH 5.5. When a pH gradient was applied, raising the pH from two (5.5 to 7.5) to three units difference (5.5 to 8.5) did not yield an increase in initial uptake rates, indicating that the pH value on the inside of the proteoliposomes was not rate-limiting for glutamate transport by GltT (data not shown). This result contrasts with the pH dependence of $DctA_{Bs}$, where the internal pH is rate limiting (chapter 3). SstT does not couple serine/threonine transport to protons, and is still active at a pH of 5.5. The highest activity was found around a pH of 6.5-7.0, as found by Kim *et al* (61).

Eukaryotic glutamate transporters are strictly potassium ion dependent, and require outward transport of a potassium ion to reset the transporter. Glt_{Ph} does not require potassium for substrate transport (82, 90). Depleting proteoliposomes harboring GltP or SstT from potassium did not influence the initial transport rates (figure 5/6). To exclude the effect of trace quantities of potassium on the inside of the liposomes, we added a large excess of potassium on the outside of liposomes with GltP, thereby creating a large inward potassium gradient. Again we observed no influence on [^{14}C]glutamate transport by GltP. We conclude that bacterial glutamate transporters do not transport potassium ions. On the amino acid level, prokaryotic transporters lack for instance a conserved glutamate residue deemed essential for potassium coupling (E404 in GLT-1 from rat (59)).

When we applied a negative membrane potential by using an outward-facing potassium ion gradient and the potassium selective ionophore valinomycin, we observed an increase in initial transport rates for all three proteins that was dependent on the size of the potassium ion gradient (only GltT shown). From this experiment we conclude that a net positive charge is transported across the membrane, and that transport is electrogenic. Transport in the absence of valinomycin thus leads to charge build-up on the inside of the membrane, which will limit further uptake of substrate.

Next to the cation import that is strictly coupled to substrate influx, an uncoupled chloride conductance has been observed for various eukaryotic glutamate transporters (29, 110). This chloride conductance was also observed by Ryan and Mindell for both Glt_{Ph} and GltT (91). They showed that inward gradients of chloride or nitrate (another permeable anion) could partially alleviate charge build-up generated by substrate transport, and thus stimulate transport rates while a gradient of gluconate (a non-permeable anion) could not. We aimed to repeat the experiments performed by Ryan and Mindell, and extend the experiments to GltP and SstT. We were unable to observe any effect of the addition of chloride to the external solution in our transport experiments for any of the three proteins. Our results obtained for Glt_{Ph} also differed from the results obtained by Ryan and Mindell. We did not see any differences between gluconate and chloride supplemented buffers, irrespective of lipid composition, protein:lipid reconstitution ratio, buffer composition or substrate concentration (data not shown). The only significant effect we observed was upon the addition of nitrate to the external buffer. In the absence of nitrate initial rates of aspartate transport by Glt_{Ph} were approximately 1.5 fold lower than in the presence of nitrate. However the apparent stimulation of transport by nitrate was independent of the magnitude of the inward nitrate gradient; a similar stimulation of transport was observed when the nitrate concentration was equal on both sides of the membrane. Furthermore, an outward facing nitrate gradient did not lead to a decrease in initial transport rates. We conclude from our data that external nitrate stimulates the transport activity of the protein, but that there is no evidence that nitrate is transported through Glt_{Ph}. Thus far, we have not been able to explain differences in our results compared to those of Ryan and Mindell, even though we have performed some of the experiments in the Mindell lab, in order to ensure identical experimental conditions.

In conclusion our data show that there are both differences and similarities between bacterial and eukaryal glutamate transporter homologues. The availability of the Glt_{Ph} crystal structure provides us with a template to interpret the differences in substrate and cation binding in structural terms. The precise substrate to cation stoichiometry of most prokaryotic transporters is still unknown, but we recently showed that Glt_{Ph} cotransports 3 sodium ions with aspartate (chapter 4). The prokaryotic homologues provide an excellent platform to study substrate and cation selectivity in transporters, because a wide range of different substrates and cations is transported by the various members.

Acknowledgements

We would like to thank Joe Mindell and Emma Compton for their hospitality. This work was supported by a *vidi* grant from the Netherlands Organisation for Scientific Research (NWO) to D-J.S.